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RNA helicase A is not required for RISC activity ☆

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ABSTRACT

It has been shown that siRNAs can compete with each other or with endogenous miRNAs for RISC components. This competition may complicate the interpretations of phenotypes observed through siRNA-mediated knockdown of genes, especially those genes implicated in the RISC pathway. In this study, we re-examined the function of RNA helicase A (RHA), which has been previously proposed to function in RISC loading based on siRNA-mediated knockdown studies. Here we show that reduced RISC activity or loading of siRNAs was observed only in cells depleted of RHA using siRNA, but not using RNaseH-dependent antisense oligonucleotides (ASOs), suggesting that the impaired RISC function stems from the competition between pre-existing and newly transfected siRNAs, but not from reduction of the RHA protein. This view is further supported by the findings that cells depleted of a control protein, NCL1, using siRNA, but not ASO, exhibited similar defects on the loading and activity of a subsequently transfected siRNA. Transfection of RHA or NCL1 siRNAs, but not ASOs, reduced the levels of endogenous miRNAs, suggesting a competition mechanism. As a positive control, we showed that reduction of MOV10 by either siRNA or ASO decreased siRNA activity, confirming its role in RISC function. Together, our results indicate that RHA is not required for RISC activity or loading, and suggest that proper controls are required when using siRNAs to functionalize genes to avoid competition effects.

1. Introduction

RNA interference (RNAi) is a commonly used technique to characterize genes by specifically degrading the target mRNAs [1]. This process is mediated by small interfering RNAs (siRNAs), 21–24 nts double stranded RNAs (dsRNAs). The double stranded siRNAs are loaded into the RNA induced silencing complex (RISC) containing Ago2. The passenger strand siRNA is then released, whereas the guide strand siRNA remains associated with Ago2 and directs the RISC to cleave the complementary substrate mRNAs (e.g., [2–4]). In addition to siRNAs, endogenously expressed miRNAs also utilize overlapping RISC components to function mainly in down-regulating gene expression either by inhibiting translation or by modulating mRNA stability [3,4].

Since siRNAs and miRNAs utilize overlapping cellular machinery for biogenesis and function, it is not surprising that these molecules can compete with each other for the binding factors, such as Ago2 in the RISC complex. Competition between co-transfected siRNAs or sequentially transfected siRNAs has been reported, leading to reduced activity for the less competitive siRNAs when co-transfected, or delayed loading of the latterly transfected siRNAs [5,6]. In addition, a recent study demonstrated that transfected small RNAs, including siRNAs and miRNAs, can globally perturb gene regulation by endogenous miRNAs most likely by competition with and saturation of the endogenous RISC pathway [7]. Several limiting factors in the RISC pathway have been proposed, including Exportin 5 and especially Ago2 [8–11]. Reduced Ago2 level has been demonstrated to increase competition between siRNAs [6]. In addition, we recently also found that transfected siRNAs can compete with endogenous miRNAs for Ago2 binding, leading to reduced levels of Ago2-associated miRNAs or even total cellular miRNA levels, and reduced protein levels secondary to impaired miRNA activity that led to increased levels of proteases targeted by miRNAs [12]. These findings show that the pre-transfected siRNAs used to reduce the transcripts of a target gene may reduce the activity of subsequently transfected siRNAs, or reduce endogenous miRNA regulation. This is particularly important when characterizing genes implicated in RISC pathway, since secondary siRNAs targeting reporter genes or reporter genes regulated by endogenous miRNAs are often used in such analyses. In these cases, it is very likely that siRNA-mediated reduction of the target transcripts can display a phenotype due to impaired RISC activity secondary to competition.

Several proteins have been implicated in RISC pathway, including MOV10, eIF6, C3PO complex, Exportin 5, and RHA [13–16]. RHA (DHX9) is a helicase protein and functions in various processes. It is able to bind single- and double-stranded RNA and single stranded DNA, and can unwind RNA–RNA and RNA–DNA duplexes [17]. This protein is implicated in transcriptional regulation and was identified as a bridging factor

Abbreviations: RHA, RNA helicase A; ASOs, Antisense oligonucleotides; RISC, RNA induced silencing complex

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between the SMN complex and RNA polymerase II, implying a role in co-transcriptional processing of RNAs [18–20]. In addition, RHA was found to associate with siRNAs and some RISC protein components including Ago2, Dicer, and TRBP [16]. RHA was proposed to function in RISC loading, as evidenced by impaired siRNA activity and siRNA association with Ago2 in cells depleted of RHA by siRNAs [16]. However, when characterizing the effects of siRNAs on the levels of endogenous miRNAs, we previously found that transfection of RHA siRNA and several other siRNAs targeting different protein-coding genes all caused reduced levels of Ago2 associated miRNAs, in a manner unrelated to loss of the target proteins [12]. These observations prompted us to re-examine the role of RHA in RISC pathway, since the previously observed defects caused by siRNA-mediated reduction of RHA transcripts on RISC activity might stem from siRNA/siRNA competition.

In this study, we reduced RHA using two different approaches, siRNA or RNaseH-dependent antisense oligonucleotides (ASOs), and found that the impaired RISC activity was not related to the loss of the RHA protein, rather, it was correlated with the presence of pre-transfected siRNAs, in a dose dependent manner. Our results indicate that RHA is not required for siRNA loading or RISC activity, and suggest that competition effects may lead to false-positive phenotypes.

2. Materials and methods

2.1. Materials

siRNAs were purchased from Applied Biosystems or Invitrogen. The ID numbers and sense sequences of the siRNAs used in this study are: RHA siRNA (s4019), 5′-GAGUGUAACAUCGUAGUAAtt; RHA-si-544, 5′-GAAGTGCAAGCGACTCTAG; NCL1 siRNA (s9312), 5′-GGAUAGUUACUGACCGGGAtt; NPM1 siRNA (S9676), 5′-CGACAAAG AUUAUCACUUUtt. The lower case letters represent deoxynucleotides that are not complementary to the target mRNAs. MOV10 siRNA (HSS106677), 5′-CCAUGUCCAUUGUAAGACCAGCUUU; PTEN siRNA (ISIS341401), 5′-AAGUAAGGACCAGAGACAA. The following siRNAs were purchased from IDT (RHA-si-544), 5′-GAAGUGCAAGCGACUC UAG; Luciferase siRNA, 5′-CGUACGCGGAAUACUUCGAUU.

2′-O-methyloxylethyl modified (2′-MOE), phosphorothioate backbone-containing RNA–DNA chimeric ASOs were synthesized as described previously [21]. The ID numbers and sequences are: RHA (ISIS230445), 5′-AATGGTGTTCGGAACATCTC; NCL1 (ISIS110074), 5′-GTCATCGTCATCCTCATCAT; MOV10 (ISIS526335), 5′-GTAACAGACTG GGTTCCGCC. The underlined nucleotides are modified with 2′-MOE. The 10 nts in the middle are deoxynucleotides. All nucleotides are linked with phosphorothioate backbone.

Antibodies against RHA (ab26271, 1:1000), NCL1 (ab13541, 1:1000), MOV10 (ab80613, 1:1000), and Ago2 (ab57113, 1:1000) were purchased from Abcam. RPL4 antibody (11302-1-AP, 1:1000) was from Proteintech. Secondary antibodies (1:2000) conjugated with HRP for mouse (172–1011) and rabbit (172–1019) were purchased from Bio-Rad.

2.2. Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% FBS, 0.1 μg/ml streptomycin, and 100 Unit/ml penicillin. Sixteen hours prior to transfection, cells were seeded at ~50% confluency in 15 cm dishes. Transfection of siRNAs was performed using 5 μg/ml Lipofectamine RNAiMax (Invitrogen), based on the manufacturer's procedure, at final concentrations as indicated in figures. Transfection of RNaseH ASOs was performed using 4 μg/ml Lipofectamine 2000 in Opti-MEM medium (Invitrogen) for 4 h, based on the manufacturer's instruction. Four hours after ASO transfection, medium was replaced with DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. 24 h after the first transfection of ASOs or siRNAs, cells were washed with PBS and re-seeded in 6-well dishes at ~50% confluency. Second siRNAs were transfected 4 h later at different concentrations using Lipofectamine RNAiMax. Four hours after the second transfection, cells were washed in plates 3 times with 1× PBS, trypsinized, and washed another 2 times with 1× PBS to remove extracellular siRNAs. Finally, total RNA or whole cell extract was prepared for qRT-PCR, western, or immunoprecipitation analyses, as indicated in figure legends.

2.3. Western analysis

Equal amount of proteins (~20 μg) prepared from test cells were separated in 4–12% SDS-PAGE and transferred to membrane. Blocking and detection of proteins with ECL was performed as described previously [22].

2.4. RNA preparation and qRT-PCR

Total RNA was prepared from HeLa cells using the RNeasy Mini Kit (Qiagen) for qRT-PCR to detect the levels of mRNAs. For miRNA or siRNA analysis, total RNA was prepared using the miRNeasy Kit (Qiagen), based on the manufacturer's instruction. qRT-PCR using TaqMan primer probe sets was performed as described previously [22]. The primer and probe sequences for different mRNAs are: MOV10: forward, 5′-ATGG TGTGGATGTGGAAGTC; reverse, 5′-AGAGTGGGAAGAGGTGAGTG; probe, 5′-TTGAACCGCAAAGAGGTGCTGAC. RHA: forward, 5′-CCACTTACTGATA CTCCTGACAC; reverse, 5′-CAGGAACACCATAGCCAGAG; probe, 5′-TGCTT TGAGAGCCAGATGTGGAGG PTEN: forward, 5′-AATGGCTAAGTGAAGAT GACAATCAT; reverse, 5′-TGCACATATCATTACACCAGTTCGT; probe, 5′-TTGCAGCAATTCACTGTAAAGCTGGAAAGG. NCL1: forward, 5′-GCTTGG CTTCTTCTGGACTCA; reverse, 5′-TCGCGAGCTTCACCATGA; probe, 5′-CG CCACTTGTCCGCTTCACACTCC. NPM1: forward, 5′-TCCTGCGCGGTTGTT CTC; reverse, 5′-GGCGGCACGCACTTAGG; probe, 5′-CAGCGTTCTTTTAT CTCCGTCCGCCT. U16 snoRNA: forward, 5′-CTTGCAATGATGTCGTAATT TGC; reverse, 5′-TCGTCAACCTTCTGTACCAGCTT; probe, 5′-TTACTCTG TTCTCAGCGACAGTTGCCTGC.

2.5. RNA immunoprecipitation

An equal amount (~150 μg protein) of whole cell extracts prepared in Buffer A [25 mM Tris.Cl pH 8.0; 5 mM $MgCl_2$; 150 mM KCl; 10% glycerol; 0.5 mM PMSF; 5 mM β-mercaptoethanol; one tablet of Protease Inhibitor Cocktail/50 ml (Roche), and 100 Unit/ml RNase Inhibitor] were incubated at 4 °C for 3 h with 30 μl Protein A beads (Roche) pre-coated with 10 μg Ago2 antibody (ab57113). After six washes with wash buffer (50 mM Tris.Cl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.1% NP-40; 20 Unit/ml of RNase Inhibitor), the co-selected RNAs were directly prepared from the beads using Tri-Reagent, dissolved in 20 μl DEPC water, and subjected to reverse transcription-qRT-PCR analysis for miRNAs or siRNAs. To control for loading, 10% of whole cell extract used in immunoprecipitation was subjected to total RNA preparation using miRNeasy kit, and the levels of U16 snoRNA were detected and used to normalize the qRT-PCR results for miRNA and siRNA.

2.6. miRNA and siRNA detection

The levels of miRNAs and siRNAs were determined using TaqMan qRT-PCR assay, based on the manufacturer's instructions with minor revision. Briefly, 5 μl total RNA (500 ng) or Ago2 co-precipitated RNAs were reverse transcribed in a 20 μl reaction containing 4 μl 5× RT buffer, 2 μl 10 mM dNTP, 1 μl RT primer specific to miRNAs or siRNA (from Applied Systems), 1.5 μl 25 mM MgCl2, 1 μl Reverse transcriptase (RMV), 0.5 μl RNase Inhibitor (Promega), and 5 μl DEPC water. The reaction was performed at 16 °C for 30 min, 42 °C for 30 min, and stopped by heating at 85 °C for 5 min. The reaction was 1:2 diluted with DDW, and 4 μl diluted cDNA was used in a 10 μl qRT-PCR reaction containing 5 μl 2× master mix (Applied Biosystems) and 1 μl primer probe mix (Applied Biosystems) specific to the miRNAs or siRNAs. The qRT-PCR program was: 95 °C for 10 min, followed by 40 cycles of

95 °C for 15 s and 60 °C for 20 s. The results were normalized to U16 snoRNA, calculated, and plotted.

The primer probe sets for miRNAs were purchased from Applied Biosystems. Assay IDs are: Let-7a (377); miR-16 (000391); miR-17 (002308); miR-21 (000397); miR-24 (000402); miR-27a (000408). Primer probe sets for PTEN siRNA, RHA siRNA, and NCL1 siRNA were customized and purchased from Applied Biosystems. The primer probe sets for RHA and NCL1 siRNAs were specific for RHA-S4019 antisense siRNA sequence (5′-aaTTACTACGATGTTACACTC-3′) and for NCL1-S9312 antisense siRNA sequence (5′-aaTCCCGGTCAGTAACTATCC-3′), respectively.

3. Results

3.1. Reduction of RHA protein does not always impair RISC activity

To ascertain if reduction of the RHA protein is the actual cause of impairment of the RISC activity as observed in a previous study [16], HeLa cells were transfected with a RHA specific siRNA or ASO. RHA mRNA can thus be reduced via different pathways, siRNA-mediated RISC pathway or ASO-mediated RNaseH pathway. 24 h after transfection, the RHA mRNA level was reduced by more than 85% in either case, as analyzed by qRT-PCR (data not shown). The RHA protein level was also dramatically reduced, as determined by western immunoblotting (Fig. 1A), indicating that similar levels of reduction of the protein were achieved through different pathways.

Next, we analyzed if RHA protein is required for RISC activity. HeLa cells depleted of RHA by treatment with siRNA or ASO for 24 h were transfected again with an siRNA targeting PTEN mRNA, at different concentrations. Total RNA was prepared 4 h after the second transfection. The activity of PTEN siRNA was detected by measuring the levels of PTEN mRNA using qRT-PCR (Fig. 1B). The IC_{50} of the PTEN-siRNA was ~0.4 nM or ~0.8 nM in control cells or in RHA-siRNA treated cells, respectively, indicating a reduced PTEN-siRNA activity in RHA-siRNA transfected cells. This observation is consistent with the previous study,

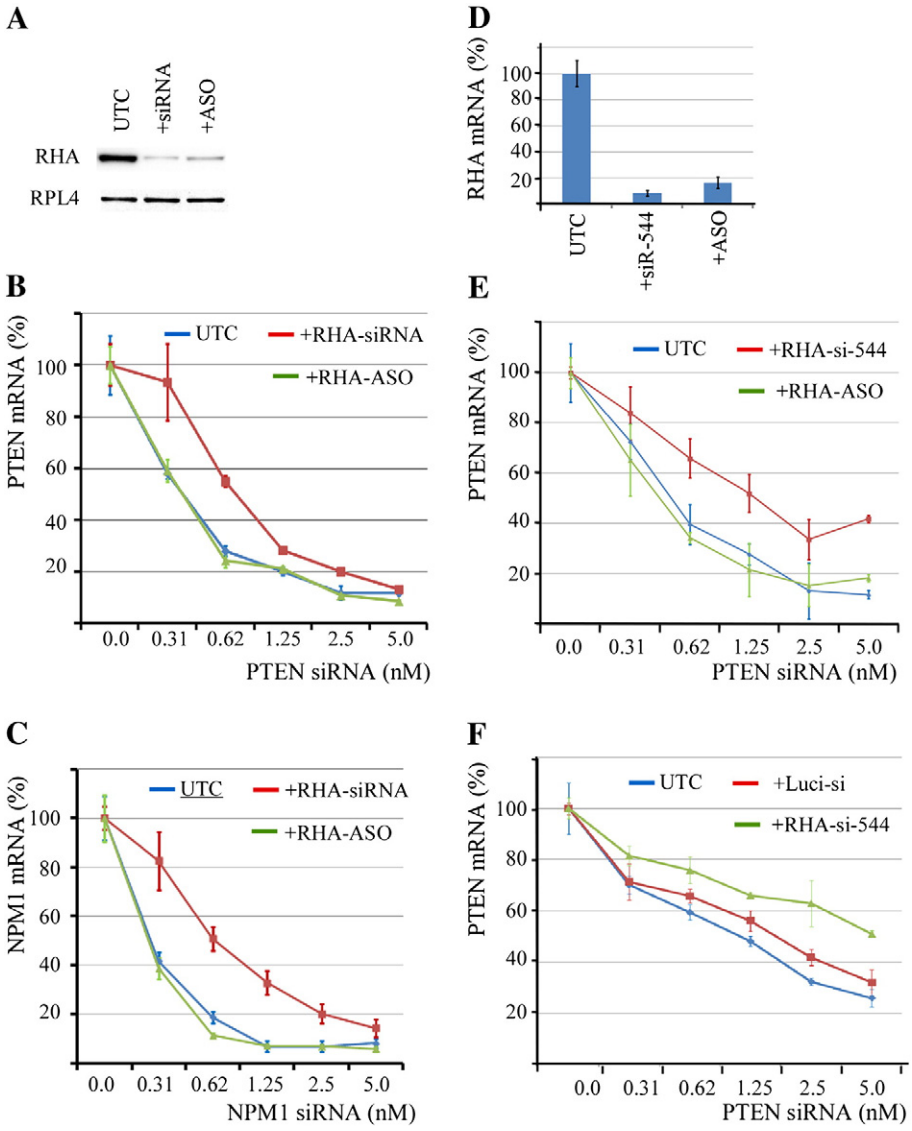

Fig. 1. RHA protein is not required for siRNA activity. A) Western analyses of RHA protein in cells treated with 5 nM RHA siRNA (+siRNA) or 50 nM RHA ASO (+ASO). RPL4 was detected and served as a loading control. UTC, cells transfected without siRNA or ASO. B) qRT-PCR analysis for the levels of PTEN mRNA. HeLa cells pre-treated with RHA siRNA (RHA-siRNA) or ASO (RHA-ASO) as used in panel A were transfected for 4 h with PTEN siRNA at different concentrations, as indicated. Total RNA was prepared and PTEN mRNA levels were detected using qRT-PCR. C) qRT-PCR analyses for the levels of NPM1 mRNA in RHA reduced cells transfected with different concentrations of NPM1 siRNAs, as in panel B. D) qRT-PCR for the level of RHA mRNA in cells transfected with 5 nM RHA-si-544 or 50 nM RHA-ASO. E) qRT-PCR assay for the level of PTEN mRNA in cells pre-transfected for 24 h with RHA-si-544 or RHA-ASO as in panel D, followed by transfection with different concentrations of PTEN-siRNA, as in panel B. F) qRT-PCR for PTEN mRNA in cells pre-treated with 5 nM luciferase siRNA (Leu-si) or RHA-si-544. The activity of subsequently transfected PTEN-siRNA was determined as in panel E. The error bars represent standard deviation from three independent experiments in all panels.

in which the reduction of siRNA activity was observed in RHA-siRNA treated cells, as compared with control cells transfected with a scrambled siRNA [16]. However, in cells depleted of RHA using ASO treatment, no defect on PTEN siRNA activity was detected, although the RHA protein level was similarly reduced by either siRNA or ASO (Fig. 1A). These results suggest that the loss of RHA protein is not the actual cause of impaired PTEN siRNA activity.

To determine if impaired activity could also be observed with other siRNAs, RHA reduced cells were transfected with siRNAs targeting Nucleophosmin (NPM1), at different final concentrations (Fig. 1C). Consistent with the results observed for PTEN siRNA, reduced NPM1 siRNA activity was again detected only in cells pre-treated with RHA-siRNA, but not with the RHA-ASO. The IC_{50} of NPM1-siRNA was comparable in control and RHA-ASO treated cells, estimated at ~0.3 nM, indicating that the loss of RHA protein itself did not impair the activity of the subsequently transfected NPM1-siRNA. However, in RHA-siRNA treated cells the IC_{50} of NPM1-siRNA was increased to ~0.6 nM, indicating that the impaired RISC activity is not unique to a specific siRNA. Together, these results demonstrate that the impaired RISC activity is not due to loss of the RHA protein, and suggest that it is very likely a consequence of competition between the pre-existing RHA-siRNA and the subsequently transfected siRNAs, as reported previously [6].

In the previous study [16], RHA was reduced using siRNAs with sequences different from those employed here, however, the impaired RISC activity is not due to unknown effects related to RHA-siRNA sequence. This view was supported by the fact that although the same siRNA as used in the previous study (RHA-si-544, corresponding to the siRNA targeting the RHA mRNA position at 544–564) caused a significant reduction of RHA mRNA, as was the case of RHA-ASO (Fig. 1D), reduced PTEN siRNA activity was only observed in the RHA-si-544 treated cells, but not in RHA-ASO treated cells (Fig. 1E), again indicating that the reduced siRNA activity does not stem from depletion of RHA protein, rather, it is due to the presence of RHA-siRNAs, in a sequence independent manner.

Interestingly, we note that in the previous study, reduced RISC activity was observed with RHA-siRNAs when compared with a control siRNA [16]. A similar effect was also detected in the current study using a luciferase siRNA as a control. The results showed that whereas the RHA-si-544 siRNA led to a significant reduction in PTEN-siRNA activity, the luciferase control siRNA only caused slight defects (Fig. 1F), consistent with the previous report [16]. These observations suggest that a functionally inactive siRNA may be less competitive for endogenous RISC components than active siRNAs. This view is supported by our previous observations that an inactive siRNA targeting a snoRNA (U16) caused less reduction of endogenous miRNAs when compared with other active siRNAs targeting endogenous mRNAs [12], and that different siRNAs exhibited different abilities to compete [5,6].

3.2. Transfection of a siRNA targeting Nucleolin can also cause similar reduction of RISC activity

Next, we reasoned that if the impaired activity of PTEN- or NPM1-siRNA resulted from competition with RHA-siRNAs for RISC components, siRNAs targeting other genes should also cause similar effects. To confirm this hypothesis, HeLa cells were first transfected with siRNA or ASO targeting Nucleolin (NCL1), a protein involved in ribosome biogenesis [23]. 24 h later, PTEN- or NPM1-siRNA was transfected and the levels of the target mRNAs were determined by qRT-PCR. As expected, the NCL1 protein, but not RHA protein, was dramatically reduced by treatment with either NCL1-siRNA or NCL1-ASO (Fig. 2A). Once again, cells pre-treated with NCL1-siRNA, but not with NCL1-ASO, exhibited reduced activity of the PTEN-siRNA (Fig. 2B) or NPM1-siRNA (Fig. 2C), indicating that the impaired siRNA activity stems from the presence of pre-transfected siRNAs and is not due to the reduction of the NCL1 protein. Interestingly, reduction in siRNA activity appears greater in NCL1-siRNA treated cells than in RHA-siRNA treated cells, although in both cases the same concentration of pre-transfected siRNAs was used (5 nM), suggesting that different siRNAs are differentially favored by the RISC components, consistent with previous reports [6,12]. Together, these results suggest that other pre-existing siRNAs can also affect siRNA activity in a way independent of target reduction, most likely due to competition between siRNAs.

3.3. Depletion of RHA protein does not affect siRNA loading

It has been reported that the RHA protein is required for RISC loading, based on the observations that the amount of siRNAs that co-immunoprecipitated with Ago2 was lower in RHA-siRNA treated cells than that in control cells [16]. However, the reduced siRNA loading may also stem from competition between siRNAs for limited RISC factors such as Ago2. To discriminate these possibilities, HeLa cells were transfected with siRNA or ASO targeting RHA, or targeting NCL1 as a control, followed by transfection of 5 nM PTEN-siRNA. As expected, the mRNA levels of RHA or NCL1 were reduced by more than 80% in cells treated with corresponding siRNAs or ASOs (Fig. 3A). Consistently, the PTEN siRNA activity was again reduced by pre-transfection of either RHA- or NCL1-siRNA, and not by ASOs (Fig. 3B). The total cellular levels of the transfected PTEN siRNA were comparable in different test cells, as detected using qRT-PCR analysis for the antisense strand siRNA (Fig. 3C), suggesting that the transfection efficiency was not significantly affected by the first transfection, in agreement with the previous observations [16].

Next, immunoprecipitation was performed using an Ago2 antibody, and co-precipitated PTEN antisense siRNA was analyzed using TaqMan qRT-PCR assay. Similar to the previous report [16], the level of Ago2-associated PTEN siRNA was much lower in RHA-siRNA treated cells, as compared with control cells (Fig. 3D), indicating that loading of PTEN siRNA to RISC was impaired in RHA-siRNA treated cells. However, the reduced siRNA loading was not due to the loss of the RHA protein, since ASO-mediated reduction of RHA had no significant effect on PTEN siRNA loading (Fig. 3D). Importantly, impaired siRNA loading was also observed in cells pre-treated with NCL1-siRNA, but not with NCL1-ASO, suggesting that the loading defect probably resulted from competition between siRNAs for binding to RISC component, especially Ago2. Note that the reduced siRNA loading was not due to decrease in the level of Ago2 protein, as no significant reduction or even a slightly increased level of Ago2 protein was observed in siRNA pre-treated cells (Fig. 3E). Together, our results indicate that impaired PTEN siRNA loading is not due to reduction of RHA (or NCL1) protein, and suggest that the pre-transfected siRNAs lead to detrimental effects on siRNA loading and RISC activity.

3.4. siRNA loading is impaired by the presence of pre-transfected siRNAs in a dose dependent manner

Competition between different siRNAs has been reported previously [5,6,24]. If this is the actual cause of the observed defects in PTEN-siRNA loading and activity, stronger defects should be observed in cells pre-transfected with higher levels of siRNAs. To test this hypothesis, HeLa cells were transfected with 0.5 or 5.0 nM RHA-siRNA for 24 h, followed by transfection with 5 nM PTEN-siRNA for 4 h. The RHA protein was reduced to similar levels by RHA-siRNA treatment at either concentration, as determined by western analysis (Fig. 4A). The transfection efficiency of PTEN-siRNA was also comparable between control and siRNA pre-treated cells, as similar levels of total cellular PTEN-siRNA were observed (Fig. 4B).

Next, the levels of Ago2-associated PTEN siRNA were determined by immunoprecipitation followed by qRT-PCR assay. Again, reduced siRNA loading was observed in RHA-siRNA transfected cells (Fig. 4C). Importantly, the loading defect was greater in cells transfected with higher levels of RHA-siRNA, as ~50% or 6% PTEN-siRNA was co-precipitated from cells pre-transfected with 0.5 or 5 nM RHA-siRNAs, respectively,

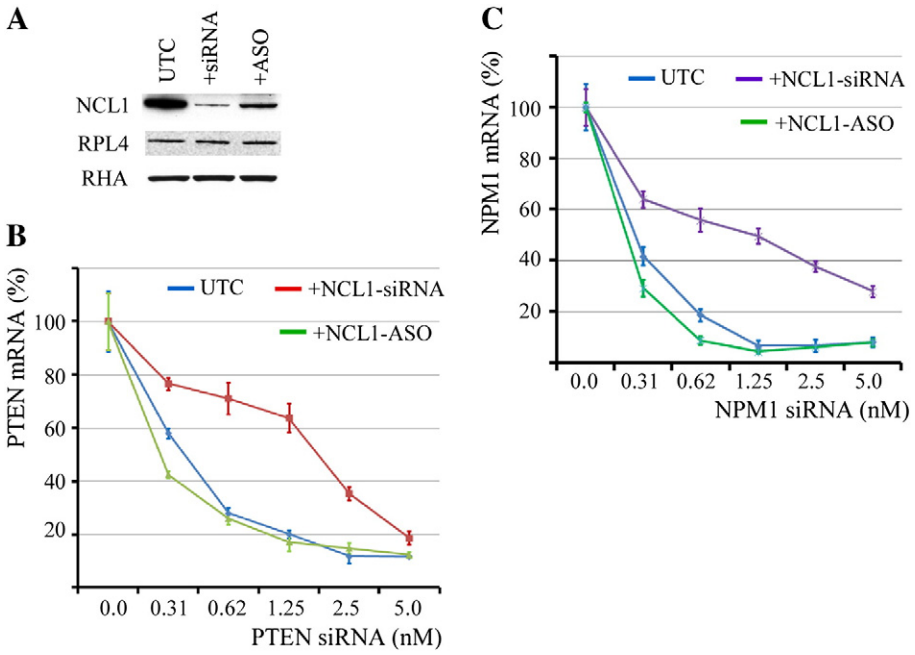

Fig. 2. Pre-treatment with NCL1-siRNA, but not ASO, also reduced RISC activity. A) Western analysis for NCL1 protein levels in cells transfected with 5 nM NCL1 siRNA or 50 nM ASO, as in Fig. 1A. RPL4 was detected and served as a loading control. B) qRT-PCR analysis for the levels of PTEN mRNA in control or different NCL1 reduced cells that were transfected again with PTEN siRNA, as in panel B. C) qRT-PCR for the levels of NPM1 mRNA in control or NCL1 reduced cells that were transfected with NPM1 siRNA at different concentrations. The error bars represent standard deviation from three parallel experiments in all panels.

relative to that in control cells. Importantly, higher levels of RHA-siRNA were co-immunoprecipitated with Ago2 from cells transfected with 5 nM RHA-siRNA as compared with that from cells transfected with 0.5 nM siRNA. These results suggest that RHA-siRNAs compete with PTEN-siRNA for Ago2 binding, in a dose dependent manner.

A similar effect was also observed in cells pre-transfected with NCL1-siRNA. The NCL1 protein was reduced to similar levels with 0.5 and 5 nM NCL1-siRNAs (Fig. 4D), and the total cellular levels of subsequently transfected PTEN-siRNA were also comparable in these test cells (Fig. 4E). Again, a stronger defect in PTEN-siRNA loading was observed in cells transfected with higher concentration of NCL1-siRNA (Fig. 4F). Opposite to PTEN-siRNA, more NCL1-siRNAs were co-immunoprecipitated from cells transfected with 5 nM NCL1-siRNA than from cells transfected with 0.5 nM siRNA, consistent with the results obtained with RHA-siRNA. Together, these data indicate that pre-transfected siRNAs can reduce the loading of newly transfected siRNAs due to competition, and support the view that the siRNA loading defects observed in the previous study were not due to reduction of the RHA protein.

3.5. Transfected siRNAs, but not depletion of the RHA protein, can also reduce endogenous miRNA levels

We recently demonstrated that the levels of endogenous miRNAs could be reduced by transfection of different siRNAs including the RHA-siRNA [12]. To determine if the RHA protein itself is involved in the accumulation of endogenous miRNAs, the protein was reduced by either RHA-siRNA or -ASO, and the levels of six miRNAs were analyzed using qRT-PCR assay (Fig. 5A). A significant reduction was detected for all the six tested miRNAs in RHA-siRNA treated cells, consistent with our recent observation. However, no significant level change was observed in RHA-ASO treated cells, indicating that the RHA protein is not required for the accumulation of these miRNAs. Consistently, the levels of Ago2-associated miRNAs were also dramatically reduced only in cells treated with RHA-siRNA, but not with RHA-ASO, as demonstrated by RNA-immunoprecipitation and qRT-PCR assay (Fig. 5B). To further confirm the view that miRNA reduction is due to siRNA/miRNA competition, HeLa cells were transfected with two different concentrations of RHA- or NCL-siRNAs, and the levels of miR-16 and miR-21 were analyzed. Indeed, a greater reduction of miRNAs was found in cells treated with higher concentrations of either siRNA (Fig. 5C., left and right panels, respectively). In addition, the functionally inactive luciferase siRNA only exhibited much less effect on the levels of miRNAs as compared with the RHA-siRNA (Fig. 5D), consistent with their effects on the activity of subsequently transfected siRNAs (Fig. 1F), further supporting the view that functionally inactive siRNAs can be less competitive than active siRNAs. Together, these results indicate that the presence of RHA (or NCL1) siRNA, but not reduction of the proteins, significantly affects the accumulation of endogenous miRNAs, due to competition with RISC components.

3.6. Unlike RHA, MOV10 protein appears to be involved in siRNA-RISC activity

To evaluate the suitability of ASO-mediated mRNA reduction in characterizing protein effects on RISC pathway and to rule out the possibility that the phenotypes of RHA reduction were unexpectedly masked by ASO treatment, we reduced MOV10 protein using either a specific siRNA or an ASO. It has been shown that siRNA-mediated reduction of MOV10 protein in human cells reduced RISC activity, as determined using a reporter gene containing EGFP whose expression was under the regulation of miR-21 [13]. The involvement of MOV10 in the RISC pathway was also demonstrated by analyzing a mutant Armitage (Arm), the *Drosophila* homologue of MOV10, which failed to silence Stellate, a gene regulated endogenously by siRNA, and failed to cleave siRNA target in an in vitro assay [25], ruling out the possibility of siRNA-competition derived effects.

The MOV10-siRNA was transfected at 0.75 or 6 nM final concentrations. As a control, a MOV10-ASO was also transfected to reduce the protein expression. 24 h after initial transfection, PTEN-siRNA was transfected at different concentrations for an additional 4 h. As expected, the MOV10 protein level was dramatically reduced by treatment with the siRNA at two different concentrations or with

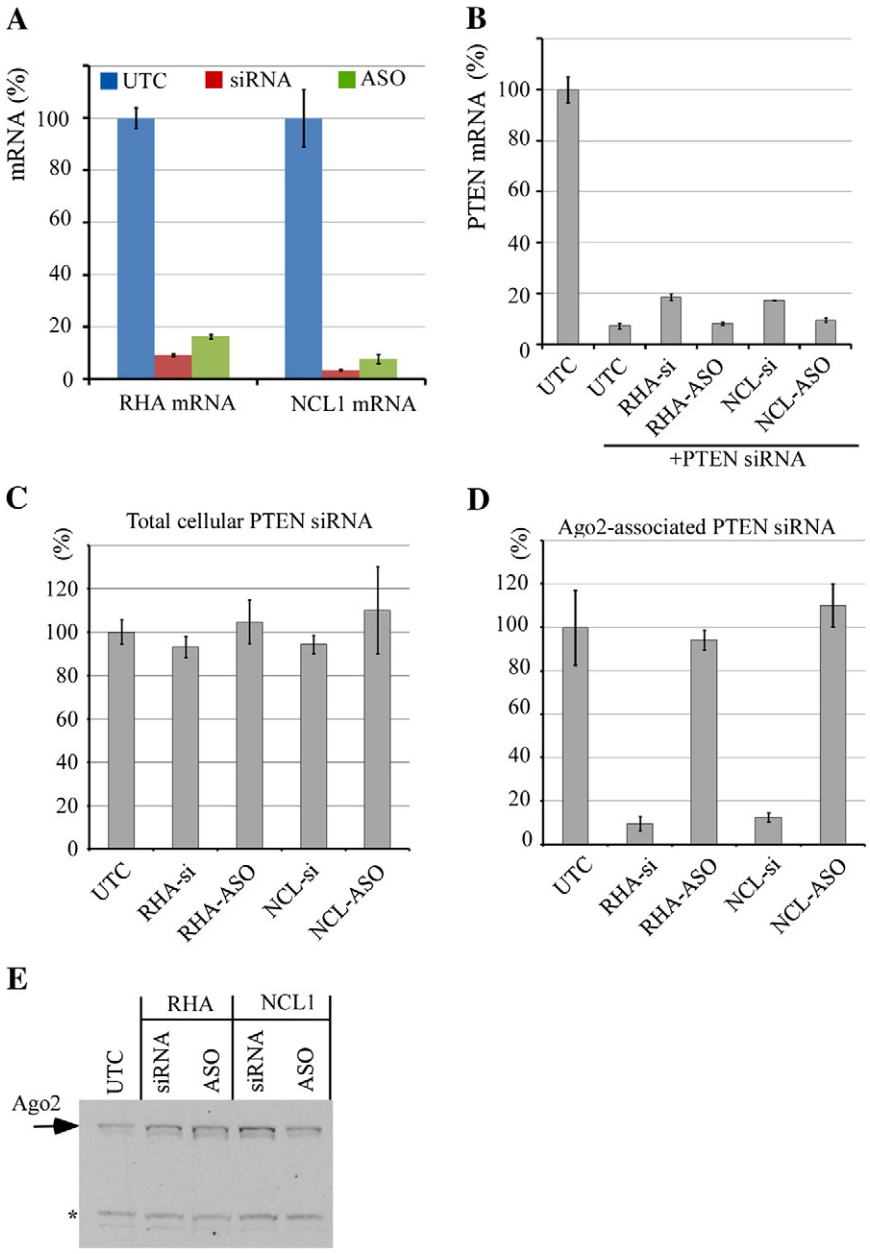

Fig. 3. Depletion of RHA does not affect siRNA loading. A) Reduction of RHA or NCL1 mRNAs by transfection of either 5 nM siRNAs or 50 nM ASOs specific to the genes, as determined by qRT-PCR analyses. UTC, cells transfected without siRNA or ASO. B) qRT-PCR results for the reduction of PTEN mRNA by transfection of 5 nM PTEN siRNA in different test cells. C) The total cellular levels of transfected PTEN siRNA appear to be comparable in different test cells as used in panel B, as determined using qRT-PCR. D) qRT-PCR for the levels of Ago2 co-precipitated PTEN siRNAs. Immunoprecipitation was performed using an Ago2 antibody from an equal amount of extracts prepared from different test cells, as used in Panel B. UTC, control cells transfected with PTEN siRNA. E) Western analysis for Ago2 protein in different test cells transfected with siRNA or ASOs. The asterisk indicates a non-specific product detected by the Ago2 antibody. UTC, control cells transfected without siRNA or ASO. The error bars represent standard deviation from three parallel experiments in all panels.

the ASO (Fig. 6A). The PTEN-siRNA activity was reduced in cells pre-treated with 0.75 nM MOV10 siRNA, as compared with that in control cells (Fig. 6B). Importantly, reduced PTEN siRNA activity was also observed in cells depleted of MOV10 by ASO treatment, indicating that reduction in the siRNA activity was not solely a siRNA competition effect and that MOV10 protein is indeed involved in RISC activity. Interestingly, a greater reduction of PTEN-siRNA activity was found in cells treated with 6 nM MOV10 siRNA than in cells treated with 0.75 nM siRNA, indicating that the reduced PTEN-siRNA activity partially attributes to the competition between the MOV10 siRNA and the PTEN-siRNA. Despite of this complexity, our results indicate that the approach of ASO-mediated protein reduction may be effectively employed to characterize the roles of proteins in RISC activity, and further support the view that RHA protein is not required for RISC activity or siRNA loading.

The potential effect of MOV10 reduction on siRNA loading was determined by analyzing the levels of Ago2 co-precipitated PTEN-siRNAs from cells pre-treated with siRNAs or ASOs. The total cellular levels of transfected PTEN-siRNA were comparable in all test cells; however, the level of Ago2-associated PTEN siRNA was much lower in MOV10-siRNA treated cells, as compared with that in control cells (Fig. 6C). Interestingly, the impaired PTEN-siRNA loading appears not to be an effect related to reduction of MOV10 protein, since no siRNA loading defect was found in MOV10-ASO treated cells, although the MOV10 protein was also dramatically reduced in this case. These results suggest that MOV10 protein is not required for association of siRNA with Ago2.

Since a stronger reduction of PTEN-siRNA activity was found in cells treated with higher MOV10-siRNA concentration (Fig. 6B), we next asked whether the impairment of RISC activity depends on the concentration of

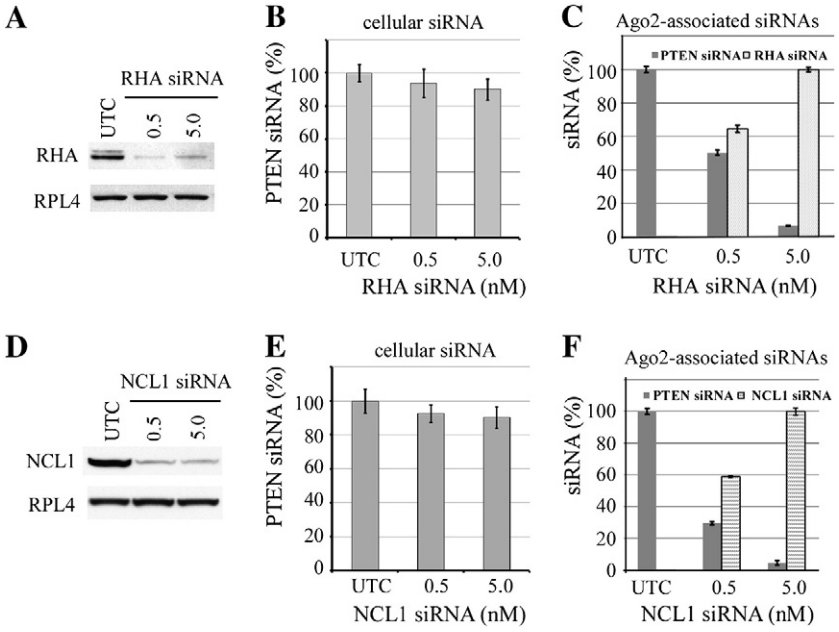

Fig. 4. siRNA loading can be affected by the pre-transfected siRNAs in a dose dependent manner. A) Western analysis for RHA protein in cells transfected with different concentrations of siRNAs. RPL4 was detected and served as a loading control. B) qRT-PCR for the cellular levels of transfected PTEN siRNA in cells pre-transfected with 0.5 or 5.0 nM RHA siRNAs. C) Less PTEN siRNA was associated with Ago2 in cells pre-transfected with higher concentration of RHA siRNA. Immunoprecipitation with Ago2 antibody and qRT-PCR for PTEN and RHA siRNAs were performed as in Fig. 3D. The relative levels of RHA siRNA were calculated based on the level of RHA siRNA co-precipitated with Ago2 from cells transfected with 5 nM RHA siRNA. D) Western analysis for NCL1 protein in cells treated with NCL1 siRNAs. RPL4 served as a loading control. E) Total cellular levels of transfected PTEN siRNA in different test cells, as in panel B. F) The relative levels of Ago2-associated PTEN and NCL1 siRNAs in different test cells, as in panel C. The relative levels of NCL1 siRNA were calculated based on the level of NCL1 siRNA co-precipitated with Ago2 from cells transfected with 5 nM NCL1 siRNA. The error bars represent standard deviation from three parallel experiments in all panels.

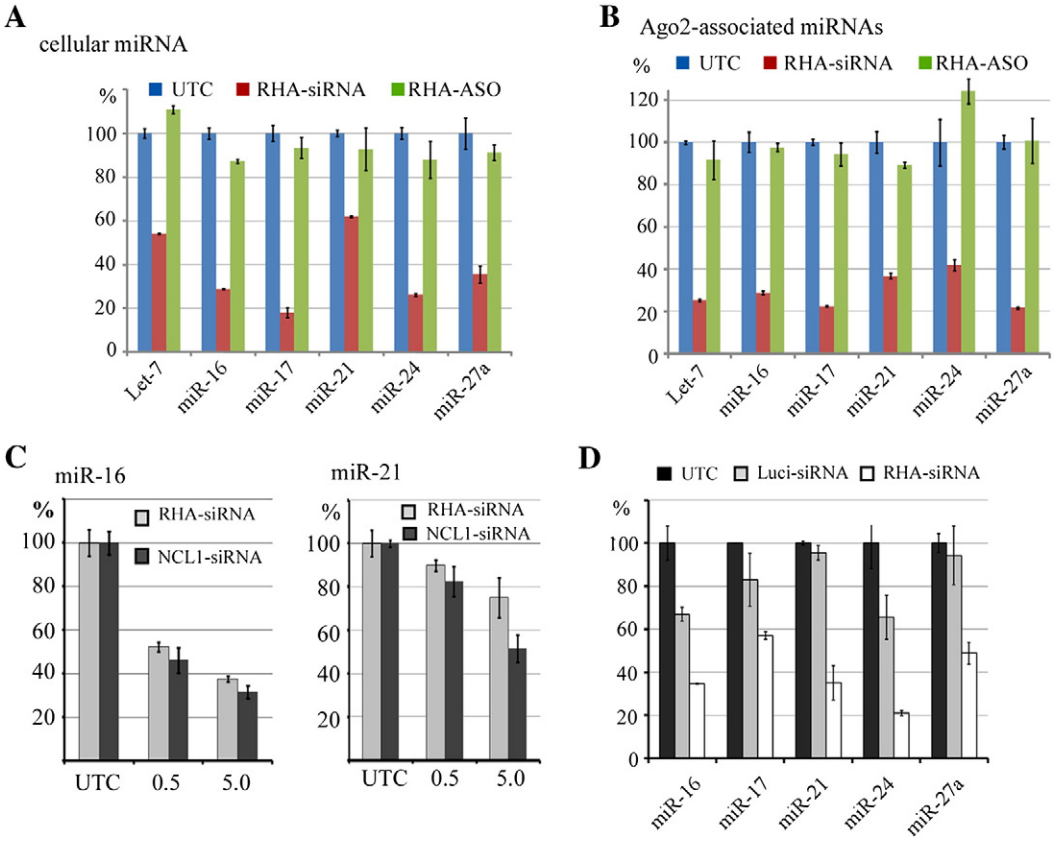

Fig. 5. The levels of cellular and Ago2-associated miRNAs can be reduced in cells transfected with siRNAs but not ASOs. A) The miRNA levels in control cells and cells depleted of RHA using 5 nM siRNAs or 50 nM ASOs. 24 h after transfection, total RNA was prepared and the levels of the miRNAs were determined using qRT-PCR, normalized to the level of U16 snoRNA, and plotted. B) The levels of Ago2-associated miRNAs reduced in cells transfected with siRNAs but not ASOs. RNA immunoprecipitated with Ago2 from extracts prepared from different cells as used in panel A were analyzed using qRT-PCR for the levels of miRNAs. C) Greater miRNA reduction can be observed in cells transfected with higher siRNA concentrations. Total RNA prepared from cells transfected with 0.5 or 5.0 nM of siRNAs targeting RHA or NCL1 was analyzed for the levels of miR-16 (left panel) and miR-21 (right panel). D) qRT-PCR for miRNA levels in cells transfected with 5 nM Luciferase siRNA (Luci-si) or RHA-siRNA for 24 h, as in panel A. The error bars represent standard deviation from three parallel experiments in all panels.

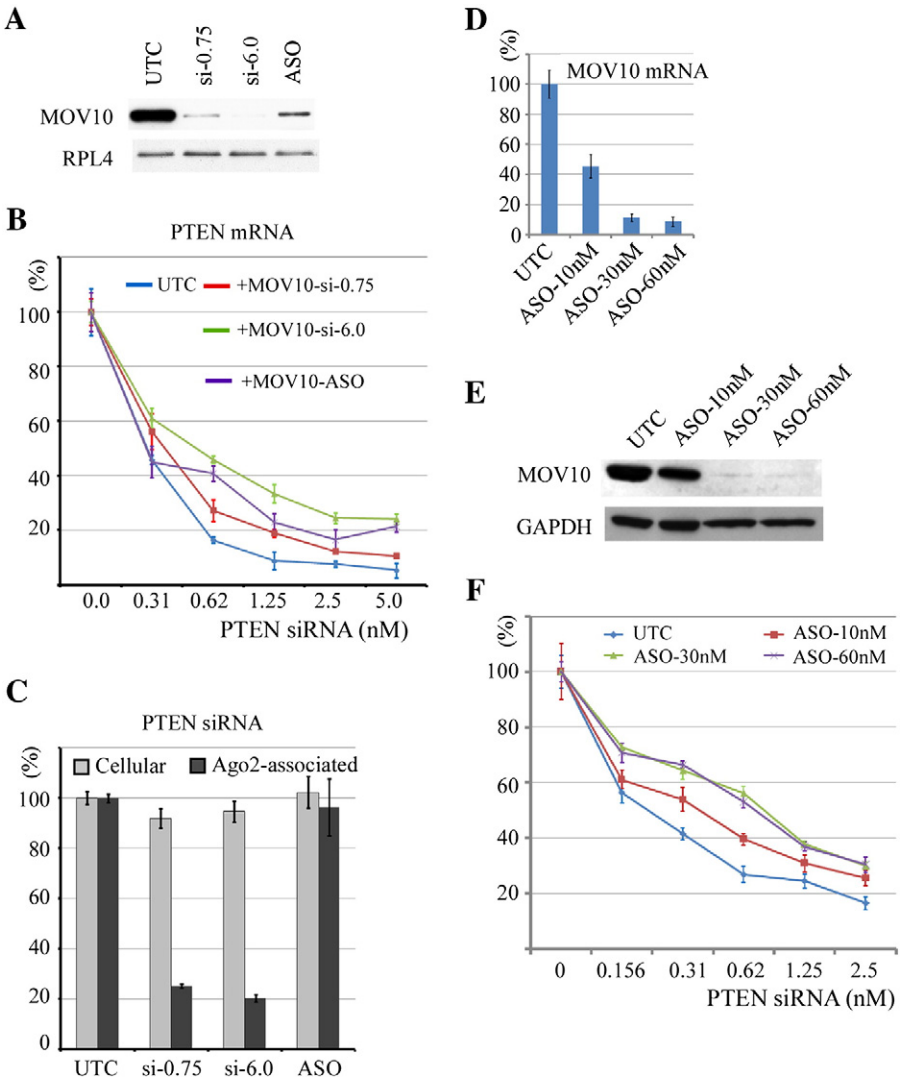

Fig. 6. ASO-mediated reduction of MOV10 impaired siRNA activity. A) Western analysis for the levels of MOV10 protein in cells transfected with 0.75 or 6.0 nM MOV10 siRNA or 50 nM ASO. RPL4 was detected and served as a loading control. B) qRT-PCR for the levels of PTEN mRNA in control cells or different MOV10-depleted cells that were subsequently transfected again with PTEN siRNA at different concentrations. C) PTEN siRNA association with Ago2 was not affected by MOV10 reduction. The levels of transfected cellular or Ago2-co-precipitated PTEN siRNA were determined using qRT-PCR. D) RT-PCR for the levels of MOV10 mRNA in cells treated for 24 h with different concentrations of MOV10-ASOs. E) Western analysis for MOV10 protein. GAPDH was detected and served as a loading control. F) qRT-PCR for the levels of PTEN mRNA in different ASO treated cells that were transfected again with different concentrations of PTEN siRNA for 4 h, as described in Materials and methods. The error bars represent standard deviation from three parallel experiments in all panels.

MOV10-ASOs, which use RNaseH mechanism. It is possible, although less likely, that the transfected oligonucleotides interact with other nucleic acid binding protein(s), which might be involved in RISC pathway. To examine this possibility, HeLa cells were transfected with different concentrations of MOV10-ASO, which is composed of hypermodified oligonucleotides that bind more proteins than canonical RNAs [26]. As expected, moderate mRNA and protein reduction was observed with 10 nM ASOs, whereas a similar and dramatic reduction (more than 90%) at both mRNA and protein levels was obtained in cells treated with 30 or 60 nM ASO concentrations (Fig. 6A and B). Consistent with the role of MOV10 in RISC activity, ~50% reduction at the protein level caused detectable impairment of RISC activity (Fig. 6F). However, a stronger defect in siRNA activity was found in cells treated with higher ASO concentrations. Importantly, no greater impairment was observed in cells treated with 60 nM ASO than in cells transfected with 30 nM ASO, in both cases the MOV10 protein was reduced to similar levels. These results, together with the observations from Fig. 1 and 2, suggest that the impaired RISC activity in MOV10-ASO treated cells is not due to unexpected effects of ASOs, rather, it is due to reduction of MOV10 protein.

4. Discussion

Competition between RISC-associated small RNAs has been demonstrated in different organisms, including mammals [7,24]. We recently found that transfection of different siRNAs in mammalian cells can alter the levels of many endogenous miRNAs, largely due to competition for Ago2 [12]. Previous work from our group also showed that different siRNAs can compete with each other, leading to a reduced activity or loading of less competitive siRNAs or subsequently transfected siRNAs [5,6]. Competition can thus lead to false-positive results especially when characterizing proteins implicated in RISC pathway. Unlike previous reports [16], in this study, we found that the RHA protein is not required for RISC activity or siRNA loading.

RHA was shown to be associated with RISC components by co-isolation using biotinylated siRNAs and by co-immunoprecipitation with different RISC pathway proteins, including Ago2, Dicer, and TRBP [16]. These findings led to a presumption that RHA might play a role in RISC pathway. The hypothesis was examined by reducing RHA using siRNAs targeting the RHA mRNA, followed by transfection of other siRNAs targeting reporter genes (GFP or CDK9) and determining

the activity of siRNAs. Reduced activity for reporter siRNAs and reduced formation of active RISC for GFP siRNAs were found in RHA-siRNA treated cells, as compared with control cells, leading to the conclusion that RHA protein functions in RISC loading [16]. Consistent with the previous observations, defects in siRNA activity and loading were also found in the current study in cells treated with different RHA-siRNAs. However, the observed defects, although reproducible, are not due to reduction of the RHA protein, since dramatic reduction of RHA by an ASO-directed RNaseH cleavage approach had no effect on siRNA activity nor loading into RISC. These findings indicate that the RHA protein itself is not required for RISC activity or siRNA loading, rather, the observed defects were derived from competition between pre-transfected RHA-siRNA and subsequently transfected siRNAs. This view is further supported by our observations that transfection of NCL1-siRNA also caused similar defects in siRNA activity and loading, however, transfection of NCL1-ASO had no such effect, although the NCL1 protein level was dramatically reduced in either case. In addition, we have found reduced RISC activities in cells pre-transfected with siRNAs, but not ASOs, targeting several different helicase proteins, including DDX30 and DDX36 (our unpublished data and [12]). These results further confirm that pre-transfected siRNAs, but not reduction of the targeted RHA or NCL1 protein, impaired the activity and loading of subsequently transfected siRNAs.

It has been shown in our previous study that eight different siRNAs, including an inactive siRNA that targets a snoRNA, all caused reduced levels of miRNAs upon transfection in an Ago2 dependent manner [12], suggesting that siRNAs that bind Ago2 might be able to affect subsequent siRNA loading or activity due to limited level of Ago2. This view is further supported by our current finding that a luciferase siRNA, which has no physiological target, also slightly reduced siRNA activity (Fig. 1F). In addition, a previous study has shown that GFP siRNA, a commonly used negative control siRNA, could reduce non-target mRNAs in a dose dependent manner, and the modulated mRNAs have target sequences homologues to the siRNA as small as eight base-pairs in size [27], suggesting that the GFP siRNA may also be incorporated into RISC complex and thus may be able to compete.

The detrimental effects of pre-existing siRNAs on the loading of subsequently transfected siRNAs are most likely due to competition for RISC components, as evidenced by the observations that higher siRNA concentrations caused stronger defects in the siRNA loading, and that transfection of either RHA- or NCL1-siRNAs, but not ASOs, reduced the levels of endogenous miRNAs. It has been shown that reducing the level of Ago2 increased competition of siRNAs [6], and that Ago2 is a limiting factor in the RISC pathway [10]. The limited Ago2 protein could be occupied by pre-transfected siRNAs, in a siRNA dose dependent manner, leading to reduced levels of available Ago2 for subsequently transfected siRNAs. This is supported by our findings that pre-transfection of higher level of RHA- or NCL1-siRNAs reduced the levels of Ago2-associated, subsequently transfected PTEN siRNAs, whereas the Ago2-associated RHA- or NCL1-siRNAs concomitantly increased (Fig. 4). Interestingly, it appears that different siRNAs may have different affinities to the RISC components, since stronger defects in siRNA activity and loading were observed for NCL1 siRNA, as compared with RHA siRNA. This is consistent with the previous observations that different siRNAs had different abilities to compete [5,6], suggesting a sequence dependent competition. It is possible that functionally inactive siRNAs may have less capacity in competition, as evidenced by our findings that luciferase siRNA, which has no endogenous target, only slightly reduced siRNA activity (Fig. 1F); and that U16 siRNA, which is inactive in target reduction, reduces miRNA levels to a lesser extent than other siRNAs that actively target endogenous mRNAs [12].

In a previous study, it was shown that competition between sequentially transfected siRNAs mainly occurred within 12 h after the first transfection [6], whereas in the current study we showed that competition still existed 24 h after initial transfection. The difference in the duration of the competitive effect is most likely due to a difference in experimental design. In the previous study the activity of the second siRNA was measured 24 h after second transfection [6], whereas in the current study it was measured 4 h after second transfection. Thus the total time during which strong competition occurred was 36 h post initial transfection in the previous study and was more than 28 h in the current study. In principal, competition could occur when the endogenous RISC component is saturated. Thus, the time slot for Ago2 to be released could vary with different siRNA concentrations and sequences, or even with different cell types. In addition, it may also stem from the differences in cellular concentrations of the first siRNA, or from sequence-dependent effects. This sequence- and activity-dependent competition of siRNAs may explain the previous observation that RHA-siRNA treated cells exhibited reduced RISC activity and loading relative to cells transfected with a control siRNA duplex [16], which may not be effectively or stably loaded into RISC prior to transfection of the subsequent siRNAs.

In contrast to RHA, we were able to confirm that MOV10 protein is required for RISC activity, since reduction of MOV10 by either siRNA or RNaseH ASO all impaired siRNA activity, suggesting that the approaches we used to characterize RHA are suitable to distinguish RISC defects. The impaired RISC activity in MOV10 reduced cells is consistent with the previous observation in *Drosophila* where the mutant MOV10 homologue, Arm, impaired RISC activity [25], ruling out the possibility of siRNA competition effects. However, stronger defects in RISC activity were observed in cells treated with higher level of MOV10-siRNA, suggesting that siRNA competition effects also partially contribute to the observed phenotypes. Interestingly, siRNA association with Ago2 was not affected by ASO-mediated MOV10 reduction, suggesting that MOV10 effects at a later stage during RISC function. This is in agreement with a previous study showing that MOV10 most likely plays a role after unwinding of duplex siRNA into single stranded siRNA [25].

Competition between siRNAs can impair the activity and loading of subsequently transfected siRNAs, raising a further concern about the specificity of the observed phenotypes in siRNA-activities when reducing a target protein. This is especially important when characterizing proteins implicated in the RISC pathway, since both the subsequently transfected siRNAs and endogenous miRNAs can be affected by the pre-transfected siRNAs. A negative siRNA is apparently not sufficient for control of specificity, since inactive siRNAs may not exhibit equal competition capacity. In this regard, a siRNA targeting an irrelevant gene might be a better control. On the other hand, conclusive interpretation should be supported by inactivating or depleting the target protein using other alternative approaches, such as ASO-mediated RNaseH cleavage method or genetic mutational analyses.

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